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Dr Ham's test revisited [editorial]

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EDITORIAL

Dr Ham's Test Revisited

IN 1937, Thomas Hale Ham made a remarkable observation: the cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) are hemolyzed when incubated with acidified normal serum.[1] This lysis appeared to be due to the activation of complement because procedures that inactivated complement abolished the reaction.[2] Because the red blood cells in no other disease appeared to share this peculiarity, this test became the *sine qua non* for the diagnosis of the disease.[3]

In fact, the phenomenon had been observed some years previously by Hijmans van den Bergh, who had acidified serum with "carbonic acid" and had noted the "striking result" that the cells of the patient with "ictere hemolytique avec crises hemoglobinuriques" were lysed, whereas normal cells were not.[4] Although he suspected that complement was the cause of the hemolysis, he could not prove it because the amount of fresh serum he added after inactivation of complement was insufficient to carry the reaction.

Both Hijmans van den Bergh and Ham noted that acidification of the patient's own serum could affect the lysis of his own cells. For many years, this was puzzling because antibody was the only known means by which complement could be activated and no evidence of autoantibodies could be detected in the autologous serum of most patients. The explanation of this paradox became apparent when Pillemer et al[5] delineated the alternative pathway of complement activation that did not depend on antibody. It was soon shown that the activated complement responsible for the lysis in acidified serum arose by activation of this alternative pathway, and this reaction remained one of the strongest arguments in favor of the alternative pathway at a time when its very existence was under considerable attack.[6]

Two questions were asked by the acidified serum test: (1) How is complement activated? and (2) Why are PNH erythrocytes lysed whereas normal cells are not?

The reactions of the alternative pathway have been carefully worked out.[7] The primary step is the fixation to the membrane of C3; this is accomplished either through the spontaneous "tick over" activation of plasma C3 through spontaneous hydrolysis of a thiolester bridge characteristic of the molecule or through the enzymatic activation of the natural molecule, again with the resultant rupture of the thiolester bond and covalent fixation to membrane entities.[8,9] Once C3b is affixed, it binds factor B, which is then activated to a serine protease after cleavage by factor D. This "convertase" complex, C3bBb, can then act as an "autocatalytic" amplification step in enzymatically cleaving C3 to C3b, thus forming evermore convertase complexes on the membrane.

This process appears to be aided by two factors. Acidification in some way not entirely delineated increases the activation of the pathway. It is not clear whether this is due to optimization of reaction conditions for one of the enzymes or for the fixation of C3b to membrane components. The optimum pH is about 6.2, the pH recommended in the performance of the acidified serum lysis test.

Furthermore, May et al[10] showed that the hemolysis of PNH erythrocytes in acidified serum could be increased by optimization of the concentration of Mg^{2+} to 0.015 mol/L If this was not done, not all of the abnormal cells were lysed and small populations of abnormal cells could not be detected. With the optimization of these factors, the test became both specific (with the exception noted below) and sensitive.

Even more intriguing is the question of what is different about PNH cells that results in their lysis in this reaction. We showed that the reaction resulted in the deposition of large amounts of C3b on the cell membrane[11] and that this was due to a greater efficiency of the convertase complex on the PNH membrane.[12] This proved to be due to the fact that PNH cells lack a membrane protein that regulates the activity of the "convertase" complexes, C3bBb and C4b2a.[13] This protein accelerates the disassembly of the bimolecular complexes and was called "decay accelerating factor" or DAF; more recently, it has been assigned the designation CD55. It was thought at first that its absence explained the sensitivity of these cells to activated complement,[14] but it was soon realized that another factor or factors must also be missing to account for the reactions that were observed.[15]

That factor is the "membrane inhibitor of reactive lysis" or MIRL (now assigned the designation CD59).[16] Holguin et al[17] showed that this protein, missing totally or in part on PNH red blood cells, regulates the activation of the membrane attack complex, which consists of C5b-9. Two groups have shown that at least one of the actions of the protein is to diminish the rate of assembly of the polymerized C9 complex[18,19] through an interaction with both C8 and C9.[20]

A third protein has been postulated, the C8 binding protein[21] or homologous restriction factor.[22] The characterization of this protein has been very difficult and it is now suspected that, in fact, it is simply a polymeric or otherwise altered form of CD59.

How CD55 and CD59 interact to bring about the protec-

tion of normal cells is made more clear by the work of Wilcox et al. They demonstrate in the accompanying report[23] that CD59 is the more important and that it may have some effect on the assembly or stability of the convertase complex as well as on the formation of polymeric C9 complexes. Whatever its actions, the replacement of CD59 to the deficient cells in PNH restores most of the protection of normal cells against the attack by complement.

The lack on the cells of PNH patients of several proteins in addition to CD55 and CD59 (erythrocyte acetylcholinesterase,[24] leukocyte alkaline phosphatase,[25] CD16,[26] CD14,[27] CD71,[28] etc) suggested a common posttranslational defect rather than genetic deletion. It was found that all of these proteins are attached to the membrane by a glycosyl phosphatidylinositol (GPI) anchor that consists of phosphatidylinositol, N-glucosamine, three mannose residues, and ethanolamine.[29] The nascent proteins were attached by their carboxyl terminus to the ethanolamine by a transamidation reaction in the endoplasmic reticulum. The defect in PNH has been thought to be due to some defect in these processes.

Most recent evidence has suggested that the defect is in the synthesis of the anchor. Stafford et al[30] have shown that PNH granulocytes have normal mRNA and synthesize a protein of aberrant size.[31] In a series of 10 patients, we have found that PNH granulocytes are unable to synthesize the complete anchor.[32] Although the exact step or steps at which the defect occurs has not been delineated, it is probably in the addition of the mannose or ethanolamine residues because these cells appear to be able to add the N-acetyl-glucosamine to phosphatidylinositol and to deacetylate it.[33] In GPI-deficient murine lymphocyte cell lines, defects at each of the steps in the synthesis of the anchor have been defined,[34] and the same may be true of PNH.

What ever the defect or defects, it is not likely to be absolute in most cases. Most patients with PNH have erythrocytes that are intermediate in their sensitivity to complement.[35] These cells uniformly bear small amounts of CD55 and CD59,[34,36] again, even small amounts of complement-protective proteins largely spare the cells premature destruction in vivo because the survival of these PNH II cells is only slightly shortened.[37]

Recent studies have suggested that PNH granulocytes deficient in CD55 and CD59 may possess CD16, the $Fc_{(gamma)}III$ receptor, another GPI-linked protein.[38] Although proof is lacking, it may be that this molecule is preferentially transamidated to the anchor because of its amino acid structure. Some investigators have examined the conditions for this transamidation and have found that a hydrophobic sequence of at least 13 amino acids is required.[39,40] Six amino acids can serve as acceptors of the amide and these varied in the degree to which they were transamidated to the anchor; the terminal residue that was most readily transamidated was serine,[41] which is the terminal residue of the mature GPI-linked form of CD16.[42] It may be that, when limited quantities of anchor are synthesized, a disproportionate amount is attached to this molecule.

If the lack of GPI-linked proteins is the fundamental defect in PNH, then we should be able to explain all the clinical manifestations by their absence. As yet, we cannot. The marked tendency to thrombosis that these patients have may be in part due to the absence of CD59. Ando et al[43] have shown that the insertion of polymeric C9 into the membrane of the platelet results in a process by which they are removed in external vesicles without lysis of the platelet. These vesicles do not keep the acidic phospholipids internalized, thus, they become the sites of prothrombinase formation and are strongly procoagulant.[44] If the CD59 of normal platelets is inhibited by antibody, more of these vesicles are formed because more nascent sites of polymerized C9 are built up. We have recently shown in preliminary data that PNH platelets form three to five times as many vesicles as normal platelets when limited activation of complement occurs. This might account for at least part of the thrombotic tendency in these patients.

The explanation of other symptoms is even more obscure. Most patients have a deficiency of hematopoiesis that is greater or lesser in magnitude. A few patients become leukemic after having the PNH for several years. These manifestations are not readily explained by what we now know about the GPI proteins and their absence in these cells.

HEMPAS

In only one other syndrome have the red blood cells been shown to be lysed in acidified serum: hereditary erythroblastic multinuclearity with an acidified serum lysis test (sometimes acronymically HEMPAS) or congenital dyserythropoietic anemia type II.[45] This syndrome is so clearly different from PNH that no difficulty in distinguishing them is apparent. It is a congenital disorder and is characterized by ineffective erythropoiesis with marked multinuclearity of the erythroblasts.[46] The red blood cells, once circulating, have a relatively normal survival.

The reactions that bring about the lysis of the red blood cells in acidified normal serum are very different from those that cause lysis of PNH cells. In this case, the lysis is mediated by an IgM antibody that is present in most normal serum that reacts with an antigen present only on the cells of patients with this syndrome; this antibody can be absorbed from normal serum by HEMPAS cells but not by normal cells.[47] The serum of patients with the disorder always lack the antibody and thus, unlike the case in PNH, lysis does not occur in autologous serum.

These cells are more readily lysed by complement when it is activated by cold agglutinin antibodies. They exhibit extraordinarily large quantities of the i antigen on the surface but often react the same as normal cells with anti-I antibodies. The increased lysis occurs despite the fact that both HEMPAS cells and normal cells may bind the same amount of antibody.[48] Careful analysis showed that this was due to the fact that much more C4 was fixed for a given amount of antibody and C1 on HEMPAS cells than on normal cells, thus accounting for the greater activation of complement. This contrasts with PNH cells on which the

fixation of C4 is normal but, because of the defects described previously, the fixation of C3 is vastly greater. This defect in HEMPAS cells closely resembles the effect of removal of sialic acid by the action of sialidase on the reactions of complement.[49]

The defect in HEMPAS has been ascribed to the inability to synthesize certain biantennary lactosaminylglycans of the hematopoietic cells. These molecules are constructed on a complex polymannose core and have N-acetylglucosamine as the first sugar of a long series of repeated aminyllactose groups.[50] The molecules terminate in a sialic acid residue. Three enzyme defects involved in the generation of these moieties have been found to result in the abnormalities characteristic of HEMPAS. In one, one of the enzymes responsible for the first N-acetylglucosamine is missing, resulting in the absence of the entire sialylated antenna.[51] In the second, there is a a deficiency of the membrane-bound form of galactosyltransferase,[52] which results in markedly attenuated antennae. In the third, the metabolism of the mannose-containing core is defective because of a defect in the gene encoding α-mannosidase II.[53] In each, there is defective glycosylation of surface membrane proteins, resulting in the loss of sialic acid from the surface.

How these defects result in the erythroblastic multinuclearity characteristic of this syndrome is not clear, and this has led to the suggestion that these defects are secondary to other dyshematopoietic defects. However, no other specific defects have been found. Furthermore, the fact that three enzyme defects that lead to the same biochemical result lead to a similar cellular abnormality suggests that these structures are important in membrane biosynthesis.

The somewhat serendipitous observation of Dr Ham has turned out to be one of great interest indeed. It has allowed the simple and accurate diagnosis of a disease of some importance. Equally important, the investigation of its basis has led to a number of seminal observations in immunology and cell biology. Rarely has so much come from such simple beginnings.

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